Isolation And Characterization of Plastic Degrading Fungi from Waste Dumping Site

SHORT SYNOPSIS

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by

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Certificate

I hereby declare that the Short Synopsis titled "**Isolation and Characterization of Plastic Degrading Fungi From Waste Dumping Site**" being submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Department of biotechnology under School of Engineering &Technology of Manav Rachna International Institute of Research & Studies, Faridabad during the academic year 2024-2025, It is a bona fide record of original work carried out under the supervision of Dr. Abhilasha Shourie, Professor, Department of Biotechnology, SET, MRIIRS and has not been submitted in full or part to any other university or institute for degree or diploma. Further, it is certified that Short Synopsis is plagiarism free and proof has been submitted.

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Isolation And Characterization of Plastic Degrading Fungi from Waste Dumping Site

Abstract

The plastic waste generated globally each year is huge and amounts to 350 million metric tons. It is now considered as a ubiquitous pollutant that is present in all environmental matrices like soil, air, water, flora, fauna, and humans. The harmful effects of residual plastic pollutant on human and environmental health are now visible making its remediation a serious challenge and priority concern. Plastics are chemically inert and resistant to degradation, take several hundred years in degradation in landfills, therefore they are highly persistent in the environment. Fungi are potential degraders of recalcitrant compounds and plastic polymers due to their capability to adhere and colonize on hydrophobic surface of plastic, penetrate the polymer through hyphae and breakdown the polymeric structure by secreting extracellular hydrolytic and degradative enzymes. Fungi exhibit remarkable ability to thrive on nutrient deficient substrates such as plastic polymer and utilize it as a carbon and energy source to support its growth and development. A number of fungi are reported to degrade various kinds of plastic waste such as *Penicillium sp., Phanerochaete chrysoporium, Trametes versicolor, Phlebiopsis flavidoalba, Aspergillus sp., Schizophyllum commune* and *Ganoderma sp.,* however there is huge fungal diversity yet to be explored for their plastic degradation potential. Despite numerous studies on fungal degradation of plastic waste, the efficiency of process has not yet been achieved and there is immense scope to improve the processes for reducing the decomposition time and increasing the decomposition percentage. This research work would aim towards exploration of novel fungal strains with high potential of plastic degradation, investigation of synergistic activities of fungal consortia that would increase the decomposition rate of plastic waste and evaluation of mechanisms involved in fungi-assisted plastic degradation.

Key words: plastic waste biodegradation, fungal degradation, hydrolytic enzymes, fungal consortia, myco-remediation

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1. Description of Broad Area

1.1 Plastic Waste- An Emerging Global Threat to Environment

Plastics are used across the globe and its usage is inevitable due to its versatility and functionality. It is inexpensive, durable and thermally stable, which makes it highly convenient and useful for consumers and highly profitable for plastic manufacturing industries. According to the report of 'Statista', a global data analysis and business intelligence platform, 400.3 million metric tons of plastic is produced each year globally, while also almost the same amount i.e. 350 million metric tons of used plastic waste is generated globally each year. Plastic waste has emerged as a ubiquitous pollutant that is now found in all environmental matrices like soil, air, water, flora, fauna, and humans. The accumulation of plastic waste into the environment has become as a serious challenge and priority concern because plastic is highly harmful for all the components of the environment. Plastic waste that is directly disposed into the environment is partially degraded to form smaller fragments or particles of the polymer of sizes ranging from few millimeters to micro- or nanometers. These polymer particles easily and rapidly enter deeper into the soil and ground water through leaching from landfill sites or percolation from wastewater or take direct entry into ocean water and sediments (Yan et al., 2024).

Unprecedented accumulation of plastic waste in environment has made the plastic exposure unavoidable for humans. The plastics enter the human body through inhalation, ingestion, and dermal contact and tend to cause cell damage, placenta rupture, inflammation, obstruction in respiratory passage, endocrine disruption and cancer (Yuan et al., 2022). Plastic waste has also caused serious impact on aquatic fauna which directly engulf them; plastic particles penetrate the tissue causing oxidative damage, generation of reactive oxygen species, and influence their growth and development. Plastic waste particles found in the environment in form of micro- and nano-plastics are also reported to bind to the other pollutants and act as a vector or carrier of these pollutants like heavy metals, plasticizers, pharmaceutically active compounds and toxicants, causing threat to vegetation, wildlife and humans (Wojnowska et al., 2022).

1.2 Types, Properties and Chemical Nature of Waste Plastics

The major types of plastics generated as waste are polyethylene terephthalate (PET), High-density polyethylene (HDPE), Low-density polyethylene (LDPE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PU) and polycarbonate (PC). Commonly used plastic PET is a grade-1 semicrystalline thermoplastic, which is most stable and is non-permeable. It is generally used in form of sheets and films to make water bottles, electronic items, and in packaging industries (Temporiti et al., 2022). It is chemically made up of terephthalic acid (TPA) and ethylene glycol and upon depolymerization it releases certain oligomers into the environment. Polyethylene polymers are made up of repeated unit of ethylene $(C_2H_4)_n$ which are chemically inert and sustainable to oxidation. HDPE is a thermoplastic that has features like excellent impact strength, light weight and high chemical resistance capacity, which make it very useful for industries like packaging and transportation. Low-density polythene is also a thermoplastic that is resistant to acids, bases and many solvents under normal conditions, and is commonly used to make bags due to high flexibility and strength (Siddiqui and Pandey., 2013). Another polymer PP is also a thermoplastic polymer but it is partially crystalline, having CH and CH_2 chains. PVC polymer is hard and sturdy than PET and PP as it is made up of repeated chloroethyl units which make it resistant to biological as well as chemical treatment. Polystyrene (PS), made of repeated unit of styrene $(C_8H_8)_n$, is firm, rigid and brittle at low temperature, has great insulation capacity and light weight. The polycarbonate is made of monomeric units of isocyanate and polyols, having chemical formula of $(C_{25}H_{42}N_2O_6)$. It is tough, has high insulation capacity and is used in automotive industry, 3D painting, aerospace and medical devices. This polymer releases toxic fumes while degradation. Polyurethane polymer is made of repeated units of bisphenol and phosgene with carbonate bonds; it is rigid in nature, abrasion resistance and has great elasticity (Srikanth et al., 2022).

1.3 Plastic Waste Treatment Methods

Mitigating plastic pollution is the need of the day. Although recycling of waste plastic is promoted as a greener method, but at the end of lifecycle of the plastic polymer its fate is only to get disposed in the environment and add to the pollution. Out of the total plastics produced, only 9% is recycled, 12% is incinerated, 10% is dumped in the oceans and 69% end up in the

landfills (Geyer et al., 2017). Moreover, not all types of plastics are suitable for recycling. Usually recycling adds to the cost resultantly the recycled product is often costlier than the original plastic, while the properties of recycled plastics also alter. Several chemical methods are also used for plastic degradation such as hydrolysis and oxidation, which are carried out under harsh conditions to breakdown the polymer and lead to the production of secondary pollutants that are further hazardous for the environment. Thermal methods breakdown the plastic under high temperature in absence of oxygen, result in production of harmful greenhouse gases like methane and cause toxicity in the environment.

Large amount of plastic waste is usually disposed in waste dumping sites such as landfills, where plastics are fragmented by weathering process into small particles of various sizes in due course of time. Plastics undergo chemical and physical change in which their polymeric structure breaks down into particles of different molecular weight, crystallinity, functional groups, mobility, bioavailability and toxicity. The mechanisms by which plastic waste is degraded in a landfill are photo-oxidative degradation, thermal degradation, hydrolysis and biodegradation by microbes. In landfills, plastic waste is dumped in form of piles which are left for several years for natural decomposition and produce 20 to 23 times higher greenhouse gases like methane into the environment and leach toxic chemicals in the soil and ground water which are generated from thermal or photo-oxidative degradation (Hidayah., 2018).

1.4 Biodegradation- A Sustainable Green Method

Due to highly persistent nature of plastics the physical, chemical and thermal methods of plastic are not sustainable as they do not lead to complete mineralization of the plastic polymer. Biodegradation is an effective method through which plastic polymer can be completely decomposed through environmentally benign biological process (Samir et al., 2022). Extensive research efforts are underway in this field, underscoring its potential as a sustainable solution for plastic waste management. Depending on their chemical composition, physical properties, thermosetting and thermostable nature and degradation capability, each of the plastic waste degrades in different time periods. Thus, the persistence of plastic strongly determines its degree of accumulation in the environment. There are several researches that report microbial degradation of these plastic waste.

The microbial degradation of plastic waste occurring in landfills can be accelerated and efficiency of degradation can be achieved with complete mineralization of plastic polymer by inoculating specifics microbial strains with high plastic degradation potential. This would require to identify the putative microbial strains and understand the microbial mechanisms underlying the process of biodegradation of plastics.

Table-1 Microbial Degradation of Various Types of Plastic Waste

S. No.	Type of Plastic	Plastic Degrading Microbes	Percentage degradation	Degradation Time	Reference
1	Polyethylene Terephthalate (PET)	*Microbial consortium of Bacillus subtilis, Aspergillus flavus, Aspergillus niger, Sarcina aurantiaca*	28.78%	60 Days	(Maheswaran et al., 2023)
2	High Density polyethylene (HDPE)	*Pseudomonas putida*	12.07%	90 Days	(Hooda et al., 2023)
		Rhodococcus ruber	11.36%		
		Pseudomonas stutzeri	8.28%		
		Bacillus cereus	8.26%		
		Brevibacillus borstelensis	8.02%		
3	Poly vinyl chloride (PVC)	*Bacillus amyloliquefaciens*	32.2%	110 Days	(Novotny et al., 2022)
		Trichoderma hamatum	29.3%		
4.	Low density polyethylene (LDPE)	*Serratia sp.*	40%	150 Days	(Nadeem et al., 2021)
		Stenotrophomonas sp.	32%		
		Pseudomonas Species	21%		
5.	Polypropylene (PP)	*Ascotricha sinuosa*	44.8%	90 Days	(Pallavi et al., 2023)

6.	Polystyrene (PS)	*Phelinus noxius*	16%	30 days	(Karimah et al., 2024)
		Leiotrametes menziesii	1%		
7.	Polycarbonate (PC)	*Pseudoxanthomonas sp.*	13.7%	30 days	(Yue et al., 2021)

**Note: Table created by author.*

1.5 Fungi as Potential Candidates for Plastic Biodegradation

Fungi occupy the status of decomposer community in an ecosystem and have huge potential to degrade recalcitrant plastic polymer through myco-remediation process. In several studies, the degradative performance of fungi has been found to be better than bacteria as the show exceptional ability to adhere and colonize on a variety of substrates- hydrophilic as well as hydrophobic, therefore can also attach on plastic surfaces, penetrate the polymer through hyphae and breakdown the recalcitrant polymeric structure by secreting extracellular hydrolytic and degradative enzymes (Corbu et al., 2023). Fungi are thus capable of growing on a variety of substrates, including nutrient deficient substrates such as plastic polymer and utilize it as a carbon and energy source to support its growth and development.

Fungal species like *Aspergillus* showed high degradation potential against PE than *Pseudomonas* species and in one month of the treatment decomposed 28% of the plastic by weight (Nasrabadi et al., 2023). Other fungal species *Lenzites betulina* and *Trametes versicolor* have been reported to produce hydrolytic enzymes such as laccase, ligase and manganese peroxidase to accelerate the degradation of plastic polymer by 40% (Cui et al., 2021; Maheswaran et al., 2023). A number of fungal genera are reported to degrade various kinds of plastics such as *Penicillium, Zalerion*, *Lasiodiplodia*, *Papiliotrema*, *Aspergillus, Fusarium and Pleurotus.*

Literature Review

Fungi play important role in biodegradation of plastic waste as they possess high degradative potential towards recalcitrant compounds. Fungi secrete extracellular hydrolytic enzymes which cause disruption of bonds and disintegration of polymeric structure of plastic, leading to its breakdown into monomeric form (Pathak and Navneet, 2017). A study was conducted to demonstrate the biodegradation of starch blended polyvinyl chloride films in laboratory as well in open environment using fungi *Phanerochaete chrysoporium*. Decrease in molecular weight was recorded in PVC film from 80275Da to 78866Da using gel permeation chromatography and release of CO_2 (7.85g/l) was studied by respiro metric method. *P. chrysoporium* was found to be a suitable fungus for bioremediation of PVC plastic and was proposed to be used in bioremediation of waste plastics from environment (Ali et al., 2014). The analysis of plastic polymer biodegradation has been done by using various analytical techniques like SEM, TGA, XRD, FTIR and microbial performance has been examined by growth studies, biofilm formation and enzyme assays (Bhatia et al., 2014). Degradation of LDPE by *Curvularia lunata, Alternaria alternate, Penicillium simplicissimum and Fusarium specie*s was studied by (Sowmya et al., 2015). Ninety genera of bacterial and fungal strains were investigated for their plastic degradation capability and *Bacillus megaterium, Pseudomonas sp.* and *Azotobacte*r were found to degrade different plastic polymers efficiently. Fungal strains isolated from soil *L. fusiformis* and *B. cereus* were reported to effectively degrade polyethene (Hussein et al., 2015; Shahnawaz et al., 2016). The biodegradation of plastic waste can be observed by changes in properties such as reduction in molecular weight, change in mechanical strength, surface changes and deterioration of polymer into fragments (Pathak and Navneet, 2017). There are several studies that explain the attachment of fungi on the lignin of wood, however, the formation of fungal biofilm on plastic surface and its role and mechanism of deterioration of the polymer structure is less studied (Paco et al., 2017). The hydrophobic nature of polymer is favorable for the formation of biofilm by the microbes which increases the plastic permeability for microbial digestion (Hodgson et al., 2018; Coppock et al., 2019).

Muhonja et al., 2018 claimed that fungi are better degraders of plastic than bacteria while experimenting with fungal strain of *Aspergillus oryzae* A5 and *Bacillus cereus* giving plastic

degradation rate of 53% and 0% respectively. Thirty-three species of wood decaying fungi were isolated and identified with the help of sequencing of their internal transcribed spacer (ITS) region of rDNA. Tendency of fungi to degrade the LDPE sheet was observed by incubation in Czapek -Dox broth in which plastic pieces were added, and *Phlebiopsis flavidoalba, Schizophyllum commune* and *Phanerodontia chrysoporium* were found to actively degrade the polyethylene. (Perera et al., 2021). White rot fungi use ligninolytic enzymes as major degradation mechanism that specifically act on complex compounds with lignin-like structure to solubilize and transform them in less toxic compounds. Peroxidase and laccases were reported to be major enzymes that actively biodegrade xenobiotic contaminants (Kathiravan and Gnanadoss, 2021). In a study, 31 species of mushrooms were tested for their ability to decompose plastics such as polyethylene, polystyrene, and polyethylene terephthalate (PET) over a three-month period. Upon analysis, only four fungal species demonstrated significant plastic weight loss. These top-performing species were further cultivated to identify the most effective strains for plastic degradation. The results highlighted that *Aspergillus* and *Ganoderma* species were particularly effective in breaking down PET polymer. This discovery points to the potential of these fungal strains in contributing to environmental safety by mitigating plastic pollution through biodegradation (Choi et al., 2024).

Research highlighted various microbes from marine environments, such as *Pseudomonadota, Bacteroidota, Bacillota*, and *Cyanobacteria,* for exhibiting exceptional potential in plastic degradation. The study delves into the enzymatic mechanisms involved in the degradation process, including the secretion of specific enzymes. Moreover, it reviews how different environmental factors influence the degradation process to better simulate in situ conditions. The study also emphasizes future prospects for plastic biodegradation research, underlining the need for more extensive exploration to address the plastic pollution problem effectively (Lv et al., 2024). Ascomycetous species, including *Aspergillus, Candida, Fusarium, Penicillium, Chrysoporium,* and *Trametes Versicolor,* were identified as pParticularly effective in utilizing plastic waste as a carbon source. These fungi exhibit hydrophobic interactions with the plastic surface, forming biofilms during enzyme secretion (Khatua et al., 2024).

Research Gaps

1. Decomposer fungal diversity in soil is huge, however, only few genera and species have been studied for their plastic degradation capability.
2. Efficiency of plastic degradation not yet been achieved using bacterial or fungal cultures.
3. Plastic degrading enzymes from fungi have not been completely characterized until now and mechanisms of degradation is not fully understood.

Problem Statement

Plastic is highly resistant to biodegradation due to which it persists for long duration in soil and water where it is disposed, causing pollution and environmental hazards. It is imperative to explore novel, effective and sustainable methods and processes for biodegradation of plastic waste. Fungi are efficient members of the decomposer community in an ecosystem that have the capacity to thrive on recalcitrant material such as macro and micro plastic substrates. In recent years, studies have reported numerous species of fungi that can degrade different types of plastics, yet there is considerable scope for identification of novel genera and species with plastic biodegradation potential. The satisfactory efficiency of the process of microbial degradation of plastic has not yet been achieved and there is immense scope to improve the processes for reducing the decomposition time and increasing the decomposition percentage. Synergistic activities of fungal consortia can be explored that would increase the decomposition rate of plastic waste.

The fungal processes involved in breakdown of plastic waste have not been fully understood such as how the fungal inhabitation on plastic surface improves the hydrophilicity of plastic polymer and how the fungi decompose high molecular weight polymer to low molecular weight oligomers and monomers by utilizing it as a source of nutrition. The mechanisms by which fungi degrade plastic waste should be deeply investigated in order to apply the fungal system for treating the plastic pollution.

Research Objectives

1. To investigate plastic degradation potential of native fungal strains found in a waste dumping site.
2. To explore synergistic activities of fungal consortia for efficient degradation of plastics.
3. To identify and characterize fungal enzymes involved in plastic degradation.

Research Methodology

Sample Collection

Plastic waste samples dumped in landfill sites will be collected in sterile silicon containers through random sampling method and will be stored at 4 °C for future use.

Isolation Of Fungi from Plastic Waste Samples

Fungi will be isolated from surfaces of the plastic waste samples and cultures will be maintained on Malt extract agar medium. The agar plates will be prepared by adding 15 g of agar to the 1 L liquid medium. Broth medium culture experiments will be carried out in 250 mL and 500 mL flasks, at 26 °C with the rotation speed of 150 rpm. Bacterial growth will be inhibited by antibiotic streptomycin added to the minimal salt medium. Fungal colonies will be selected and repeatedly transferred to fresh MEA plates until pure isolates are obtained. The single colonies/ pure isolates will be stored in glycerol at −80 °C for further use.

Screening of Plastic Degrading Fungi

Screening of plastic degrading fungi will be done on minimal salt medium (MSM) having the composition KH_2PO_4 (1.0 g/L), Na_2HPO_4 (1.5 g/L), NH_4Cl (2.0 g/L), $CaCl_2 \cdot 2H_2O$ (0.1 g/L), KCl (0.15 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), $ZnSO_4 \cdot 7H_2O$ (0.01 g/L), and $MnSO_4 \cdot H_2O$ (0.001 g/L). The isolates will be cultivated in MSM supplemented with sterilized plastic discs/ particles at 26°C. Fungal mycelium will be taken from pure culture obtained after isolation and will be inoculated in MSM (both plates and broth) containing plastic pieces particles.

Morphological Identification of Fungi

Morphological identification of Fungi will be done on the basis of macroscopic and microscopic examination of isolated fungal strains. Macroscopic evaluation will involve observation of the colony features like color, shape, texture and size.

For microscopic evaluation, the slides of isolated fungi will be prepared by mounting a small segment of the mycelium and staining it with lactophenol cotton blue, and will be observed under a compound microscope to visualize the hyphae and mycelia (Razaq et al., 2018).

Molecular Identification of Fungi

Genomic DNA will be extracted from the mycelia of the fungal isolates, and the internal transcribed spacer (ITS) region will be amplified through PCR using the universal primers for identification of fungi on the basis of ITS region. The ITS gene sequences of the fungal isolates will be aligned with the sequences retrieved from the database through CLUSTAL W for phylogenetical analysis (Gong et al.,2023).

Determination of Weight Reduction of Plastic by Fungi

The plastic discs will be weighted before inoculation and after fungal treatment at an interval of every 10 days. Prior to weighing, the discs will be washed thoroughly with surfactants followed by several washings with distilled water to remove the attached fungal mycelia. The discs will be dried on filter paper and the percentage of the weight loss will be calculated by –

$$\% \text{ weight loss} = \text{initial weight} - \text{final weight} / \text{initial weight} \times 100$$

Atomic Force Microscopy (AFM) And Scanning Electron Microscopy (SEM)

The fungi treated plastic will be subjected to Atomic Force Microscopy and Scanning Electron Microscopy to study the topography and morphology of plastic disc and to test weather fungal mycelium action could result into any micro level changes or any cracks pits on surface while decomposing.

FTIR Analysis

FTIR analysis of fungi treated plastic will be performed to detect the chemical composition as well as functional group change in the disc after fungal plastic interaction by analyzing its infrared spectrum.

UV-Visible Spectroscopic Analysis

The appearance of characteristic plastic degradation products in the medium after fungal treatment of plastic will be analyzed on the basis of absorbance maxima obtained after scanning through entire UV-VISIBLE region. This will also enable to detect the production of chemical compounds in the degradation process and analyze any new product or change in concentration of the solution due to degradation process.

Laccase Assay

Laccase activity will be assayed with taking guaiacol as a substrate, where 2mM of guaiacol will be added to sodium acetate buffer (10 mM, pH -5.5) to prepare the reaction mixture and 2 ml of enzyme will be added. The mixture will be incubated at 28^{o} C for 15 minutes and absorbance will be observed at 450 nm in the spectrophotometer (Desai et al., 2011)

Lignin Peroxidase Assay

Lignin peroxidase enzyme activity will be estimated using veratryl alcohol (3,4 dimethyloxybenzylalcohol) as a substrate. The culture supernatant (2 ml) as an enzyme source will be mixed with 10 ml of citrate buffer (0.1M, pH 5.0) and the substrates 2.0 ml veratryl alcohol and 500Um hydrogen peroxide will be added. The reaction mixture will be incubated at 18^{o}C for 10 minutes and absorbance will be observed at 310 nm in the spectrophotometer (Shi and Wang., 2019; Husaini et al.,2011).

Xylanase Assay

Xylanase activity will be analyzed by using xylan as a substrate where 1% of xylan will be dissolved in 0.05M citrate phosphate buffer (pH 5.0) and 2 ml of culture supernatant will be added. The reaction mixture will be incubated at 55^{o}C for 10 minutes and absorbance will be observed at 540 nm in the spectrophotometer (Lowe et al., 1987; Sridevi and Charya., 2011)

Cutinase Assay

The cutinase assay will be done using p- nitrophenyl butyrate as a substrate, where 0.56mL p-nitrophenyl butyrate will be mixed with 0.05M citrate phosphate buffer (pH 5.0) and 2 ml of culture supernatant will be added as an enzyme source. The reaction mixture will be incubated at 37^{o}C for 15 minutes and absorbance will be observed at 405 nm in the spectrophotometer (Macedo et al., 2005).

Manganese Peroxidase Assay

The manganese peroxidase enzyme will be assayed using Mangnese solphate ($MnSO_4$) as a substrate, where $MnSO_4$ will be mixed with 0.05M sodium tartrate buffer (pH 5.0) and 2 ml of culture supernatant will be added as an enzyme source. The reaction mixture will be incubated at room temperature for 10 minutes and absorbance will be observed at 238 nm in the spectrophotometer (Husaini et al., 2011; Vrsanska et al., 2016).

Analysis of Plastic Waste Degradation Products

Degradation products of plastic waste that will appear in the medium after fungal treatment will be analyzed through HPLC, LC-MS and GCMS. Identification will be done on the basis of mass spectral libraries and standards, and quantification will be done through calibration curve using standards and peak area from chromatogram.

Expected Outcome of The Research

This research work will identify novel fungal strains with plastic waste degradation capability which could be used as promising candidates for myco-remediation applications. Investigation of synergistic activity of different fungal genera and species will disclose the potential of consortia in plastic polymer disintegration, which in future would enable to prepare robust bioinoculants for landfill applications to accelerate the plastic waste degradation. Studies on cellular and molecular mechanisms used by fungi will lead to deeper understanding of processes imperative for plastic waste degradation such as inhabitation of fungi on plastic surface, changes in hydrophilicity properties of the polymer, biofilm formation, utilization of plastic as carbon source and complete mineralization of plastics.

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